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DCM-Causing Mutation E361G in Actin Slows Myofibril Relaxation Kinetics and Uncouples Myofibril Ca^{2+} Sensitivity from Protein Phosphorylation
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Previous experiments using the unloaded in vitro motility assay have shown that the myofilament Ca^{2+}-sensitivity is modulated by phosphorylation by PKA of troponin I (Ser 22 and 23) and that the DCM-causing mutation *ACTC* E361G uncouples this relationship. Here, we determine whether this also happens in heart muscle myofibrils producing isometric force. Myofibrils were isolated from wild-type and *ACTC* E361G mouse hearts. We know that TnI and MyBP-C phosphorylation levels are high in normal mouse heart (1.4 molPi/mol TnI). To reduce the phosphorylation level of TnI and MyBP-C, mice were injected with a high dose of beta-adrenoceptor agonist propranolol which reduced phosphorylation of both TnI and MyBP-C by 70-80-%. Myofibrils were isolated from propranolol-treated and untreated mouse hearts. Then we measured contraction in single myofibrils with a Ca^{2+}-jump protocol using a range of Ca^{2+}concentrations. The maximum isometric force was the same for phosphorylated and dephosphorylated WT and *ACTC* E361G mouse myofibrils. The duration of the linear slow phase of relaxation was longer (1.4-1.8 fold) and the rate of the fast phase was reduced (1.1-1.4 fold) for the *ACTC* E361G TG compared to WT. The Ca^{2+}-sensitivity of isometric force was increased 1.4-fold (p=0.021) when WT myofibrils were dephosphorylated. In contrast the *ACTC* E361G Ca^{2+}-sensitivity was not altered by dephosphorylation. This pattern was also apparent in the rates of relaxation. Comparing dephosphorylated WT *vs* WT, the duration of the linear slow phase was longer and the rate of the fast phase was reduced but dephosphorylation did not change the kinetics of E361G myofibril. Thus the *ACTC* E361G mutation fully uncoupled Ca^{2+} sensitivity from the level of TnI and MyBP-C phosphorylation in intact myofibrils as we have seen with IVMA.

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There is a Limit to the Changes in Myofilament Ca^{2+}-Sensitivity due to Myopathies
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We have investigated Ca^{2+}-sensitivity changes in cardiac and skeletal muscle due to mutations or phosphorylation of troponin I (TnI) using the *in vitro* motility assay.
In normal heart, TnI phosphorylation alters myofibrillar Ca^{2+}-sensitivity and increases the speed of Ca^{2+}-dissociation (lusitropic effect). When end-stage heart failure samples with a reduced level of phosphorylation were looked at, the average Ca^{2+}-sensitivity change was x2.6 ± 0.24.
Eight dilated cardiomyopathy (DCM) mutations have been looked at in all components of the thin filament (Actin E361G, TnT R141W and ΔK210, TnI K36Q, TnC G159D and Tm E40K, E54K and D230N). Although they all have differing effects on Ca^{2+}-sensitivity (some increased it and some decreased it) the average Ca^{2+}-sensitivity change was x2.0 ± 0.16.
The actin E99K hypertrophic cardiomyopathy (HCM) mutation causes an increase in Ca^{2+}-sensitivity of x2.5 but a literature search looking at 21 other HCM mutations in every sarcomeric protein has found an average Ca^{2+}-sensitivity change of x1.8 ± 0.13.
A wide range of skeletal myopathies were looked at which either caused a gain of function (*TPM2* ΔK49, ΔE139, *ACTA1* K326N and *TPM3* ΔK7) or loss of function (*TPM2* E117K, R91P and *TPM3* A4V, L100M and R167C). The mean changes of Ca^{2+}-sensitivity were x1.8 ± 0.3 and x2.7 ± 0.4 respectively.
It is remarkable that mutations causing a range of different striated muscle myopathies have such a similar and limited effect on myofilament Ca^{2+}-sensitivity and that phosphorylation of troponin I regulates Ca^{2+}-sensitivity over the same range. We have found in DCM and HCM that Ca^{2+}-sensitivity is uncoupled from the level of TnI phosphorylation and tends to the Ca^{2+}-sensitivity characteristic of phosphorylated wild-type muscle at all phosphorylation levels. It is tempting to speculate that the 2-fold change represents a transition between two activation states of the thin filament.

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Effect of the Troponin I Restrictive Cardiomyopathy Mutation R145W on Protein Expression in Murine Murine Hearts
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Restrictive cardiomyopathy (RCM) is a relatively rare type of cardiomyopathy but is assocated with a high incidence of sudden cardiac death (SCD). The R145W mutation in troponin I have been shown to be associated with RCM and in patients with this mutation SCD has been found to occur. A transgenic mouse model of RCM expressing troponin I R145W showed increased calcium sensitivity of force development and impaired muscle relaxation. Isobaric tags for relative and absolute quantitation (iTRAQ), a non-gel-based technique used to quantify proteins from different sources in a single experiment, was used to determine the differences between hearts from 3 month old transgenic control mice (mice expressing human cardiac troponin I) and R145W mice (mice expressing human troponin I with the R145W mutation). Four hearts each from R145W and wild-type transgenic mice were proteomically investigated and several signaling pathways were found to be affected by the R145W mutation. Increases in enzymes involved in ATP production (such as NAD(P) transhydrogenase), kinases (calmodulin dependent protein kinase II beta), intermediate filament proteins (desmin), de-ribosylating enzyme (ADP-ribosylarginine hydrolase), proteolytic enzymes (tripeptidy peptides II), glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase), and stress related proteins (inducible heat shock protein 90aa). At the proteolytic level the activity of the proteasome was decreased in hearts from R145W transgenic mice. No change in the protein expression levels of the 20S protein PSMA6 or the 19S protein Rpt1 was observed in these hearts. These results suggest that the RCM mutation R145W is associated with significant and complex changes in cardiac protein expression.

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Mutations in Cardiac Myosin Binding Protein - C Associated with Hypertrophic Cardiomyopathy Alter Structure, F-Actin Binding and Phosphorylation
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A common cause of hypertrophic cardiomyopathy (HCM) is mutations and modifications of the sarcomeric protein cardiac myosin binding protein C (MyBPC). MyBPC binds to the F-actin thin filament via its N-terminus, the myosin thick filament backbone via its C-terminus, and reversibly binds to the myosin-S2 neck region (when de-phosphorylated). The functional consequences of nine hypertrophic cardiomyopathy mutations within the N-terminal phosphorylation region, domains C1C2 were determined. Of the nine HCM mutations investigated, only two (Tyr237Ser and His257Pro) showed decreased solubility and altered folding, compared to the wild-type C1C2, as measured by circular dichroic spectroscopy. Phosphorylation did not alter the secondary structure content of C1C2. Wild-type C1C2 binds F-actin with 1:1 stoichiometry and micromolar affinity (Kd ~ 7 μM), using a co-sedimentation assay. All mutants exhibited weaker binding to actin, except for Asp228Asn and Arg326Gln, which bound similarly to WT. Protein kinase A phosphorylation activity was assessed in four HCM mutants, located in close proximity to the phosphorylation sites. The Gly278Glu mutation inhibited phosphorylation, Arg326Gln and Leu352Pro increased the rate, while no difference was observed for the Gly279Ala mutation. The order of protein kinase A phosphorylation of C1C2 was determined to be: phosphorylation of Ser284 (Site B), followed by Ser275 (Site A). The third and final phosphate was added at Ser304 (Site C), at a much slower rate (0.015 min 1). The effects of hypertrophic cardiomyopathy mutations on C1C2 structure and function were consistent with the mild phenotype associated with these mutations.

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Electron Microscopy and 3D Reconstruction of Regulated Thin Filaments Decorated with Cardiac Myosin Binding Protein C
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Myosin binding protein C (MyBP-C) is an accessory protein of striated muscle thick filaments and a modulator of cardiac muscle contraction. Defects in the cardiac isoform, cMyBP-C, cause heart disease. cMyBP-C comprises eleven 10 kDa immunoglobulin- and fibronectin-like domains, numbered C0-C10 from the N-terminus, and a MyBP-C-specific motif between C1 and C2. In vitro studies show that, in addition to binding to the thick filament via its C-terminal region, cMyBP-C can also interact with actin via its N-terminal domains, modulating actin motility. Neutron scattering and 3D EM reconstructions of F-actin decorated with N-terminal fragments of cMyBP-C suggest that cMyBP-C binds to subdomain 1 of actin close to the low Ca^{2+} binding site of tropomyosin. This suggests that cMyBP-C might modulate thin filament activity by physically interfering with tropomyosin regulatory movements on actin, a possibility strengthened by solution kinetics observations (White and

Harris, Biophys. Soc. abstracts, 2012). To determine directly whether cMyBP-C binding affects tropomyosin position, we carried out 3D reconstruction of negatively stained thin filaments (containing F-actin, tropomyosin and troponin) decorated with the N-terminal fragment containing domains C0 to C2. Clear decoration was obtained under a variety of salt conditions. 3D reconstructions suggest that under most conditions cMyBP-C does not displace tropomyosin from its low Ca^{2+} position, although under certain conditions some shift of tropomyosin did appear to occur. At high Ca^{2+}, there was little effect on tropomyosin position. The results suggest that cMyBP-C may modulate thin filament function by physically competing with tropomyosin for its low Ca^{2+} site on F-actin.

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Calcium-Calmodulin Competes with Actin for Binding to the M-Domain of Cardiac Myosin Binding Protein-C

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The regulatory M-domain of cardiac myosin binding protein-C (cMyBP-C) binds to myosin, actin, and to calmodulin when calcium is present (i.e., calcium-calmodulin), but it is unclear whether binding of all three ligands is independent or if binding interactions are competitive. Here we investigated whether calcium-calmodulin (Ca-Cam) binding to the M-domain affected the ability of the M-domain to bind to actin using cosedimentation binding and calmodulin-sepaharose pull-down assays. Results of actin cosedimentation binding assays showed that Ca-Cam significantly reduced specific binding of a recombinant protein containing three N-terminal domains of cMyBP-C (i.e., C1-M-C2, referred to as C1C2) when 10 μM calmodulin was present in the presence of calcium (pCa 3.0). In the absence of calcium (at pCa 10.0) calmodulin had no effect on C1C2 binding to actin. Increasing Ca-Cam concentrations to achieve higher molar ratios with respect to C1C2 further reduced the amount of C1C2 that bound to actin. Conversely, in calmodulin-sepharose pull-down experiments, binding of C1C2 to calmodulin was only modestly reduced in the presence of increasing concentrations of F-actin. Taken together, these results indicate that binding of Ca-Cam can compete with actin for binding to the M-domain. These results suggest a potential mechanism whereby the functional effects of cMyBP-C binding to actin can be regulated by calcium. This work supported by NIH HL080367.

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Structural and Functional Studies of Phospholamban-Sarcolipin Chimeras

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The sarcoplasmic reticulum (SR) is a calcium storage organelle in muscle cells that contains a calcium pump (SERCA) required for calcium reuptake and muscle relaxation. The activity of SERCA is regulated by the small integral-membrane subunits phospholamban (PLB) and sarcolipin (SLN). PLB is present in cardiac and smooth muscle, while SLN is found in skeletal muscle and the atria of the heart. Consequently, the regulatory mechanisms imposed by PLB and SLN have clinical implications for the treatment of heart disease. There is significant sequence homology in the transmembrane regions of PLB and SLN suggesting a similar mode of binding to SERCA; however, SLN has a unique and highly conserved C-terminal tail (27RSYQY) that is lacking in PLB. The structural differences in the luminal domains between these two proteins could be responsible for the subtle differences in their regulation of SERCA. We have functionally characterized alanine mutants of the C-terminal tail of SLN using co-reconstituted proteoliposomes that mimic the SR membrane. We found that Arg27 and Tyr31 are essential for SLN function. To further study the role of the luminal tail of SLN we also tested the effect of a truncated variant of SLN (Arg27stop) as well as chimeras of PLB consisting of the wild-type sequence with the five luminal residues of SLN added to its C-terminus. The Arg27stop form of SLN resulted in loss of function, while the PLBtail chimeras resulted in super-inhibition with characteristics reminiscent of the SERCA-PLB-SLN ternary complex. These functional results are being correlated with structural studies by cryo-electron microscopy of SERCA in complex with the PLBtail chimeras. Based on our results, we propose that SERCA inhibition by SLN is encoded in the C-terminal tail, and that the functional properties of SLN are transferred to PLB in the PLBtail chimeras.

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Characterization of Cardiac Adiponectin Post-Translational Processing and Secretion

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Adiponectin (ADN) is primarily secreted from adipocytes, and serum concentrations negatively correlate with obesity, diabetes, and several other metabolic diseases. More recently, secretion and autocrine actions of cardiac ADN have been described. The purpose of this study was to characterize the post-translational modifications undergone by ADN monomers in cardiac and other mammalian, and their association into trimers, hexamers, and larger multimers (>18-mers) during secretory pathway transport. Adiponectin-flag adenovirus (Ad.ADNf) was used to overexpress the protein in mammalian cells and primary adult rat cardiomyocytes. Intracellular and secreted adiponectin were analyzed in cell extracts and 24 h supernatants. Native oligomeric adiponectin within these cellular systems was analyzed by immunofluorescence, while composite monomers and multimers were examined by SDS-PAGE and immunoblotting. Intracellular cardiomyocyte ADN accumulated in junctional SR puncta, an early ER subcompartment, consistent with current views of the cardiac secretory pathway, whereas it co-localized with classical ER markers in HEK and COS cells. Cardiac ADN was secreted as a collection of polymers comprised of a single relatively unmodified protein monomer, similar to that secreted from cultured epididymal fat. Roughly half of the protein was secreted in 24 h. In contrast, ADN from HEK cells secreted polymers comprised of ADN monomers with slightly greater modification. Minor changes in response to treatments with tunicamycin suggest that some portion of both the intracellular and secreted protein monomers undergo N-linked glycosylation. HEK cells alone exhibited significant ability to cleave ADN after its secretion. Among all mammalian cells, both intracellular and secreted ADN were comprised of distinct patterns of protein polymers. Similarities between monomeric and polymeric structures in secreted ADN and effects on adenosine monophosphate-kinase from different mammalian cells support the possibility that ADN serves both endocrine and autocrine actions for fat and heart, respectively.

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Na/K ATPase Affects Respiration Kinetics and Provides Evidence for Intracellular Diffusion Restrictions in Permeabilized Rat Cardiomyocyes

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Restrictions in the intracellular movement of adenosine nucleotides play an important role in cardiac energetics. In the work leading up to this project we confirmed that strong functional coupling exists between some ATPases and mitochondria. Such coupling is evidence of restricted diffusion in the cell. In addition our results revealed a previously unknown feedback mechanism showing that endogenous PK and a fraction of ATPases are tightly coupled. The localization of the diffusion restrictions grouping ATPases with ATP production and to what cellular structures they can be attributed to remains unknown. To address this issue we inhibited cellular ATPases one by one and analyzed their impact on respiration kinetics. The aim of this work is to investigate if sarcolemma Na/K ATPase (NKA) is or is not coupled to endogeneous PK.

We found that NKA affects cellular energetics inducing a 45% drop in ATPase activity initiated by 2mM ATP. To investigate the effect further we performed a set of six experiments used on the control cells to compare the kinetic parameters. We found that for NKA inhibited cells Km(ADP) was significantly higher and Km(ATP) significantly lower than in the control case. Vmax for both ADP and ATP were lower for NKA inhibited cells than in the control case. We also saw a different response in respiration to PEP indicating a change in coupling between ATPases and endogeneous PK. We then analyzed this data using seven mathematical models with different compartmental specifications. This work presents the experimental results and our analysis of this data using these models.

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Proteomic Analysis of Akita Mice Reveals 9 Proteins Altered during Early Stages of Diabetic Cardiomyopathy

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Cardiovascular disease is the leading cause of diabetic morbidity and over 10% of patients with type 1 diabetes (T1DM) die before they are 40 years old. This study utilized Akita mice, a murine model with T1DM progression analogous to that of humans. Diabetic cardiomyopathy in Akita mice presents as diastolic impairment as early as 3 months of age and significant cardiac atrophy by 5 weeks. Hearts from recently diabetic mice (5 weeks) were analyzed with label free proteomics to identify proteins which may play a critical role in the pathophysiology of diabetic cardiomyopathy (n=3). At this early stage, 9 proteins were differentially expressed in diabetic mice: GANC, PLEKHN1, COLIA1, GSTK1, ATP1A3, RAP1A, ACADS, EEF1A1, HRC, EPHX2, and PKP2 (gene names). A recent study demonstrated that deletion of EPHX2,